Cell Specific Transfer of CD39-Mediated Thromboprotection

Honors Research Thesis

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by
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ABSTRACT:

Ectonucleotide triphosphate diphosphohydrolase-1 (ENTPD-1; CD39), a transmembrane enzyme expressed on platelets, leukocytes, and endothelial cells, is capable of hydrolyzing the prothrombotic purinergic signaling molecules adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine monophosphate (AMP), which is then converted to adenosine by ecto-5'-nucleotidase (CD73). Through this mechanism of action, CD39 and CD73 are capable of affecting ADP-dependent platelet activation and recruitment necessary for propagation of a growing thrombus following vessel injury. Previous work by our laboratory has shown that transgenic over-expression of human CD39 (hCD39; CD39-TG) in mice results in greatly prolonged time to vessel occlusion compared to wildtype (WT) littermates. This previous work did not specify whether CD39 over-expression on the cellular components of the blood or that on the endothelium was responsible for the protection against thrombus formation. In this study, we propose that the CD39-mediated thromboprotection results from over-expression of CD39 on bone marrow-derived cellular components of the blood. In addition, we show that it is possible to transfer this protection between genotypes by utilizing both bone marrow transplantation from an animal over-expressing CD39 to a wildtype animal and by infusion of platelets from a transgenic to a wildtype animal.

INTRODUCTION:

According to data from the United States Center for Disease Control, cardiovascular disease is the leading cause of morbidity and mortality in the United States and in the Western World.[1] A large portion of these deaths result from myocardial infarction (MI/heart attacks) and cerebrovascular ischemic incidents (strokes) caused by coagulation of blood and the formation of an occlusive thrombus. These occlusive thrombi form in response to vascular injury and restrict the flow of oxygen and nutrient-rich blood to downfield tissue, which can lead to death of the tissue if blood flow is not promptly resumed. In response to cellular injury of vessels and exposure of the sub-endothelial matrix, various signaling cascades converge and result in a local increase purinergic signaling molecules, specifically the pro-thrombotic and pro-inflammatory molecules adenosine triphosphate (ATP) and adenosine diphosphate (ADP).[2] These have been shown to activate platelets, a cellular component of the blood and one of the key mediators of thrombus formation, via the $P2Y_1$ and $P2Y_{12}$ receptors.[3,4] Once activated, platelets undergo a shape change and recruit other platelets to the site of injury by releasing ADP.[5,6] This ADP can then activate additional platelets, leading to a feed-forward cascade working to propagate the growing thrombus.[6] Because of this phenomenon, the ectoenzyme ectonucleotide triphosphate diphosphohydrolase-1 (ENTPD-1; CD39) is in a unique position to modulate platelet activation and thus thrombosis.

CD39 is a 70 – 100 kDa transmembrane protein expressed on endothelial cells and various blood cellular components, specifically platelets and leukocytes .[7,8] CD39 hydrolyzes the prothrombotic purinergic molecules adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine monophosphate (AMP).[7] Ecto-5'-nucleotidase (CD73) can then hydrolyze AMP to the antithrombotic, anti-inflammatory molecule adenosine (ADO)

(Figure 1).[9] Previous work conducted in our laboratory has shown that global transgenic over-expression of human CD39 (hCD39) in mice can prolong the time to complete cessation of flow by an occlusive thrombus in a large conduit artery following ferric chloride-induced injury of the vessel when compared with the same model of injury in wildtype (WT) mice (Figure 2).[10] As CD39 is expressed on both endothelium and blood cells, these studies did not offer insight into the specific cell populations responsible for the thromboprotection.

Here we demonstrate that circulating CD39 found on blood cells plays a larger role in the prevention of formation of an occlusive thrombus than the CD39 found on the vasculature. In addition, we have shown that it is possible to modulate thrombus formation in wildtype mice by altering the levels of CD39 found on their circulating cells via bone marrow transplants and transfusions of cells purified from whole blood.

METHODS:

Transgenic Mice:

Transgenic mice over-expressing hCD39 were generated and graciously supplied by our collaborators.[11] The human CD39 transgene is expressed from the mouse H-2K^{b} promoter resulting in global expression of CD39 in these mice. CD39-TG mice were backcrossed for more than 10 generations onto the C57-Black 6 background and compared with littermate controls. The investigations described conform to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by The Ohio State University Institutional Animal Care and Use Committee.

In Vivo Arterial Thrombosis:

Ferric chloride ($FeCl_3$)-induced carotid artery thrombosis was used for these experiments.[12] WT or CD39-Tg mice were anesthetized with ketamine (55 mg/kg body weight) and xylazine (15 mg/kg body weight). Atropine (0.05 mg SC) was administered to reduce airway secretions. Animals were intubated and ventilated with room air (tidal volume 250 µl, 150 breath/min) with a mouse respirator (Harvard Apparatus, Holliston, MA). Rectal temperatures were maintained at 37^{o}C by a thermo-regulated heating pad. The left common carotid artery was gently dissected, a flow probe placed on the artery (MA0.5PSB; Transonic Systems; Ithaca, NY) and blood flow measured with a pulsed doppler flow system. After obtaining baseline flow recordings, carotid artery injury was induced by application of filter paper saturated with 10% $FeCl_3$ solution on the adventitial surface proximal to the flow probe for 3 minutes. The flow as a percent of baseline and the time to thrombotic occlusion (blood flow of 0 ml/min) was measured from the placement of the $FeCl_3$ saturated filter paper. The operator was blinded to the animal genotype and any drug treatment during all experiments. Animals were re-anesthetized as needed and at each hour under anesthesia, animals were administered normal saline intraperitoneally.

Ablation of Bone Marrow:

Mice to receive bone marrow transplants were subjected to treatment with ultraviolet (UV) radiation from an RS-2000 Biological Irradiator (RAD-Source, Suwanee, GA) in order to ablate native bone marrow. Twenty-four hours before transplant was to occur, mice were placed inside the irradiator for 5 minutes and received 100 cGy per minute. After 4 hours, mice were

subjected to a second dose of radiation for 4 minutes (100 cGy per minute).[13] Mice were immediately placed on Baytril water treatment to help prevent infection.[14]

Bone Marrow Isolation and Transplant

Donor mice were sacrificed under sedation of ketamine (55 mg/kg body weight) and xylazine (15 mg/kg body weight). The long bones of the hind leg were then removed and stripped of connective tissue before being snapped at the knee. The epiphysis were removed with surgical scissors and a 27 gauge needle was used to flush 3 mL of RPMI buffer (CellGro, Manassas, VA) through the long bones to harvest the marrow. Bone marrow was spun at 300x g for 5 minutes at 4 degrees Celsius. Supernatant was discarded and pellet was resuspended in 10 mL of 1x red blood cell (RBC) lysis buffer (BioLegend, San Diego, CA) for 5 minutes, after which 10 mL of RPMI buffer was added and the solution was centrifuged at 300x g for 5 minutes at 4 degrees Celsius. Pellet was resuspended in RPMI with 5% (w/v) fetal bovine serum (FBS) to give a final concentration of 1 x 10^6 to 5 x 10^6 cells per 100 μL of buffer.[13,14]

Mice received bone marrow transplant approximately 24 hours after last dose of radiation was received. Mice were placed in a restraining device which allowed access to the tail. 100 μL of cell-RPMI solution was injected via the tail vein with a 28 gauge U-100 insulin syringe (Becton Dickinson and Company, Franklin Lakes, NJ).

Visualization of Thrombus Formation In Vivo:

For visualization of thrombus formation, mice were injected intravenously (via the tail vein) with 5 μL of a 100 μM solution/gram of body weight of the cationic lipophilic dye 3,3'-dihexyloxacarbocyanine iodide ($DICO_6(3)$) ten minutes prior to application of $FeCl_3$.[15] The

carotid artery was continuously video recorded using a Leica M165 FC fluorescent steromicroscope equipped with Hamamatsu ORCA-R^2 Digital CCD camera for 30 minutes.

Platelet Isolation and Injection:

Donor mice were anesthetized with ketamine (55 mg/kg body weight) and xylazine (15 mg/kg body weight). Blood was then collected via closed chest cardiac puncture with a 23 gauge syringe into a 3 mL syringe containing 200 μL Acid-citrate-dextrose.[16] Whole blood was centrifuged at 300x g for 8 minutes.[17] Platelet rich plasma (PRP) supernatant was aspirated and mixed with 1 mL Tyrode's Buffer containing 1 mM $CaCl_2$ and 2 μL of 0.5 mg/mL prostaglandin (PGE-1).[16,17] Erythrocyte-rich pellet was discarded. If the PRP appeared to have a reddish hue, it was determined that the blood cells had hemolyzed and sample was discarded. PRP-Tyrode's solution was centrifuged again at 300x g for 4 minutes. Platelets were then washed by aspirating 1 mL of supernatant and adding 1 mL of fresh Tyrode's buffer with calcium and 2 μL of 0.5 mg/mL PGE-1.[17] Resulting solution was centrifuged at 300 X g for 4 minutes. Supernatant was discarded and platelet-rich pellet was resuspended in 100 μL sterile phosphate-buffered-saline (PBS). If visualization of clot was desired, $DIOC_6$ dye was mixed with platelet solution and then injected via the tail vein with a 28 gauge U-100 insulin syringe (Becton Dickinson and Company, Franklin Lakes, NJ) 15 minutes prior to application of $FeCl_3$.

Flow Cytometric Analysis:

Whole blood was obtained via tail bleed of WT or CD39-TG mice. All antibodies were obtained from EMFRET Analytics GmbH & Co. KG (Eibelstadt, Germany) and eBioscience (San Diego,

CA). Fluorescence-activated cell sorting (FACS; LSRII, Becton Dickinson and Company, Franklin Lakes, NJ) was used with antibodies to hCD39 in order measure the level of CD39 on leukocytes to determine if blood was "transgenic" or "wildtype" regardless of genotype of animal from which it was obtained. Antibodies to CD45 were also used as a control to ensure cells being analyzed from hCD39 were blood cells derived from bone marrow. Negative controls were used to inquire if there were differences between the CD39-TG and WT animals at baseline.

Data Analysis:

The results of experiments were analyzed by several statistical methods using standard software (GraphPad Prism, version 4.0). Results were expressed as mean ± standard error of the mean. For comparison between 2 groups, significance was determined by unpaired Student *t* test. For comparison of multiple groups, multifactorial ANOVA with post-hoc comparison of the means with Bonferroni correction used to determine statistical significance. For all evaluations, probability values of $p < 0.05$ were considered significant.

RESULTS:

Transplant of hCD39-Over-expressing Bone Marrow resulted in production of hCD39-over-expressing blood cellular components

In order to ensure successful engraftment of bone marrow transplant and subsequent production of genotypically correct cells, complete blood count (CBC) and Fluorescence-activated cell sorting (FACS) analyses were performed on whole blood obtained from animals

approximately 60 days after transplant of bone marrow. CBCs confirmed that levels of white blood cells, red blood cells, and platelets had returned to baseline levels and did not significantly differ between groups (Table 1 and Figure 3) but it was unknown if bone marrow-derived blood cells were over-expressing hCD39, which would confirm successful engraftment of CD39-TG bone marrow and subsequent production of CD39-TG blood cells. To confirm successful engraftment and hematopoesis, whole blood was obtained and centrifuged to isolate leukocytes, which were washed and then incubated with antibodies to hCD39 and CD45 in order to test for the presence of the enzyme.

Pseudo-color plots show the comparison between the fluorescence of control, unstained cells, cells which had been incubated with an antibody to hCD39, and cells which had been incubated with two antibodies, one to hCD39 and one to CD45 (Figures 4a – 4e).

Histogram plots also give the relative counts of cells with respect to phycoerythrin (PE) fluorescence (Figure 5). Control cells showed no significant difference in geometric mean of a histogram plot between the two groups (WT → CD39-TG: 7.2 ± 2.0, n=3; CD39 → TG: 8.7 ± 1.3, n=3; p = ns, Figure 5). When incubated with antibodies to CD39 and CD45, however, there was a significant increase in the geometric mean of the CD39-TG → WT group, compared to the WT → CD39-TG group (WT → CD39-TG: 183.0 ± 20.5, n=3; CD39-TG → WT: 522.7 ± 35.3, n=3; $p < 0.005$, Figure 5).

Transplant of WT Bone Marrow resulted in production of WT blood cellular components

Similar to what was described above, CBC and FACS analysis was employed to determine if successful engraftment of bone marrow had taken place in CD39-TG animals which

received WT bone marrow. No significant differences were seen when comparing the CBC results of the WT into CD39-TG bone marrow transplant group to baseline levels in WT and CD39-TG mice nor when comparing the two groups of bone marrow transplants. Pseudo-color plots of the FACS data show very little difference between the control, unstained cells and those cells incubated with anti-hCD39 (Figure 4f – 4j). Analysis of the geometric means generated by the histogram plots shows significantly less fluorescence in the WT into CD39-TG group compared to the CD39-TG into WT groups when incubated with both antibodies to hCD39 and CD45 (WT → CD39-TG: 183.0 ± 20.5, n=3; CD39-TG → WT: 522.7 ± 35.3, n=3; $p < 0.005$, Figure 5).

Transplant of hCD39-Over-expression Bone Marrow Mediates Resistance to Arterial Thrombosis

To examine the role of CD39 on blood cells and endothelium individually, bone marrow transplant experiments were performed. WT mice were irradiated and received bone marrow from CD39-TG mice, thus allowing them to maintain basal levels of CD39 on endothelium but over-express CD39 on bone marrow-derived blood cellular components such as platelets and leukocytes. Conversely, a group of CD39-TG mice were irradiated and received bone marrow from WT mice, allowing these mice to maintain over-expression of CD39 on endothelium, but WT levels of CD39 on bone marrow derived blood cellular components. After engraftment of bone marrow was confirmed via FACS analysis, $FeCl_3$ – induced vascular injury experiments were performed and the time to thrombosis was measured. The time to complete occlusion of the vessel was markedly prolonged in the WT animals which received CD39-TG bone marrow, when compared to baseline times for WT mice. (CD39-TG → WT: 102.00 ± 7.71 min, n=5;

Baseline WT: 12.69 ± 2.19 min, n = 4; $p<0.005$). In the CD39-TG mice which received WT bone marrow, the time to vessel occlusion did not differ significantly from WT experiments. (WT → CD39-TG: 13.14 ± 0.41 min, n=5; Baseline WT: 12.69 ± 2.17 min, n=5). (Figure 6).

hCD39-Over- expressing Platelets Mediate Resistance to Arterial Thrombosis

To test our hypothesis that cellular components of the blood were responsible for thromboprotection seen in the CD39-TG animals, we designed experiments that involved transfusing cells purified from a CD39-TG animal into a WT animal (CD39-TG → WT), using transfusion of WT cells into WT animals (WT→ WT) as a control. For this study, the individual cell type to be tested was platelets, due to their role in thrombus formation. WT animals, which received approximately 7 million cells isolated from the whole blood of CD39-TG animals, showed significantly prolonged time to vessel occlusion following injury of the carotid artery with $FeCl_3$ (WT baseline: 12.69 ± 2.17 min, n=5; CD39-TG → WT: 179.90 ± 34.70 min, n=5; WT → WT: 11.21 ± 1.58 min, n=5; $p <0.005$) (Figure 7).

hCD39-Mediated Resistance to Arterial Thrombosis is Calcium Dependent

A second group of platelet transfusion experiments in which calcium was omitted from the isolation and wash buffer yielded a surprising result. When calcium was omitted from the Tyrode's buffer used to isolate and wash the platelets to be transfused, the thromboprotection seen before when platelets from a CD39-TG mouse were transfused into a WT mouse disappeared and the times to thrombosis showed no significant difference from baseline WT

mice (CD39-TG → WT: 9.43 ± 0.83 min, n=4; WT → WT: 11.53 ± 0.62 min, n=4; WT baseline: 12.69 ± 2.17 min, n=5; p = ns) (Figure 8).

DISCUSSION:

Based on data collected from various experiments over the course of this project, we believe the protection resulting from over-expression of the ectoenzyme Ectonucleotide triphosphate diphosphohydrolase-1 (ENTPD-1; CD39) is primarily due to up-regulated levels of the enzyme on cellular components of the blood, not elevated levels on the endothelial cells of conduit arteries. Using bone marrow transplants, we were able to focus the over-expression from global, to more tissue specific. Wildtype mice who received bone marrow from a CD39-TG mouse began producing blood cells which tested positive for the hCD39 transgene. Conversely, CD39-transgenic animals which received bone marrow from a wildtype mouse began producing blood cells which did not show expression of the hCD39-transgene. Ideally, these experiments focused the expression to the bone marrow-derived blood cells, but not the endothelium in the CD39-TG into WT transplant mice; and in the endothelium, but not the bone marrow derived blood cells in the WT into CD39-TG transplant mice. This assumption, however, requires further investigation for validation. Recent studies have suggested that endothelial cells can originate from bone marrow progenitor cells and some groups claim to have found evidence for circulating bone marrow-derived endothelial cell progenitors.[18] Experiments are currently underway in which the aorta and carotid arteries of transplant mice are being harvested to test for levels of hCD39 via western blot analysis compared to basal levels in the WT and CD39-Tg. Regardless, our results showing a markedly prolonged time to thrombosis in the CD39-TG → WT animals, but not in the WT → CD39-TG animals strongly suggest that cellular components of the blood are primarily responsible for the thromboprotection.

Upon reaching the conclusion that over-expression of CD39 on cellular components of the blood was responsible for the prolonged time to thrombosis, our lab developed experiments to test the role played by individual cell types. Due to the time constraints of this study, we determined it would only be possible to test the role of one type of cell, and as such, we chose to focus on platelets due to their well documented role in thrombosis and hemostasis.[19] Data collected from the experiments performed suggest that CD39 expression on platelets play a key role in modulating thrombosis. Simply by infusing transgenic platelets into a WT mouse, the time to thrombosis was significantly prolonged compared to untreated mice and a control group in which WT mice which received WT platelets. An important limitation of this study is that it remains unknown whether over-expression of CD39 on blood cells and platelets is simply preventative, i.e. the thromboprotection is only seen when the treatment occurs before vascular injury. The question remains if CD39 has the potential to halt and/or even reverse the formation of an occlusive thrombus if administered after vascular injury has occurred. Our laboratory is currently exploring different methods to determine if either soluble CD39 (apyrase from potatoes) or platelets isolated from the whole blood of a CD39-TG animal can modulate thrombosis if administered after vessel injury, and if so, how long after thrombosis they may be infused as an effective thrombolytic agent.

With our conclusion that the cellular components of the blood in CD39-TG animals are responsible for protection against thrombus formation and our results that this protection is able to be transferred between genotypes via the use of one of those cellular components in CD39-TG platelets, we would like to investigate the roles that other cell types potentially play in CD39-mediated thromboprotection. Recent studies have suggested that leukocytes, specifically monocytes and neutrophils, play a significant role in thrombus formation.[20,21] To test this

hypothesis in our model and to determine in CD39 over-expression on these cells can modulate resistance to thrombosis similar to what we have shown in platelets, our laboratory is currently carrying out experiments mimicking the procedure used in the platelet transfer described previously to transfuse leukocytes from a CD39-TG mouse into a WT mouse and observe the resulting change, if any, in the time to thrombosis. Another area of interest is the presence of circulating monocyte-platelet aggregates which have been shown to indicate platelet activation.[22,23] Our laboratory would like to use FACS analysis to investigate the difference between circulating monocyte-platelet aggregates ex vivo to help determine if CD39 expression on blood cells can reduce platelet activation, and thus, monocyte-platelet aggregates.

Our result that thromboprotection transfer via platelets is calcium dependent, although interesting, may not appear to be surprising when the physical characteristics of the CD39 enzyme are considered. As a phosphohydrolase enzyme, one would expect the active site to contain a cation of some sort to stabilize the strong negative charge on the phosphate backbone. Previous studies have indeed shown that CD39 requires divalent metal cations for fully functional enzymatic activity.[24] We believe that our results confirm this to be true by showing it is possible to transfer CD39-mediated thromboprotection between animals via platelets, but only when calcium is used in isolation and purification buffer. In future studies investigating the roles of various other cell types the role of calcium will also be examined to see if the same calcium dependence holds true.

An important limitation of this study is that all of the thrombosis experiments were performed on one model. In an effort to validate our results and demonstrate that they are not method specific, experiments are underway to test the thromboprotective capabilities of CD39 in a Folts-like method of thrombosis with cyclic flow reductions.[25] As the Folts method is known

to produce platelet-rich thrombi, we believe this will serve as an excellent model to validate our argument that CD39 found on platelets modulates protection against thrombus formation.[26,27]

We believe our findings presented here hold potential for novel therapeutics in the future. As stated earlier, ADP is a prothrombotic signaling molecule and has been shown to activate platelets via interaction with the $P2Y_{12}$ receptor.[2,3] Common therapeutics to fight thrombus formation includes the drug clopidogrel, commonly known as Plavix, which acts via antagonistic interaction with the $P2Y_{12}$ receptor.[28] Although this treatment has been successful, there are patients who do not respond to clopidogrel due to genetic mutations in their $P2Y_{12}$ receptors, rendering the treatment useless.[29] Our findings presented here suggest an alternative route to preventing platelet activation and aggregation. Instead of preventing activation of platelets with receptor antagonists, our data suggests that it might be equally effective to hydrolyze the signaling molecule ADP in order to prevent receptor engagement and subsequent activation.

In the future, we would like to further investigate the role of specific cellular and non-cellular components of the blood, including leukocytes, macrophages, granulocytes and microparticles. Experiments are in progress to isolate leukocytes from whole blood obtained from CD39-TG animals and inject these cells into WT animals. We would also like to confirm our finding that over-expression of CD39 on platelets modulates resistance to arterial thrombosis by using a genetic approach in which the transgene is expressed solely on platelets. Investigators are currently working on generation of a "CD39 platelet-specific" over-expressing animal for use in our future experiments.

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Disclosures:

None.

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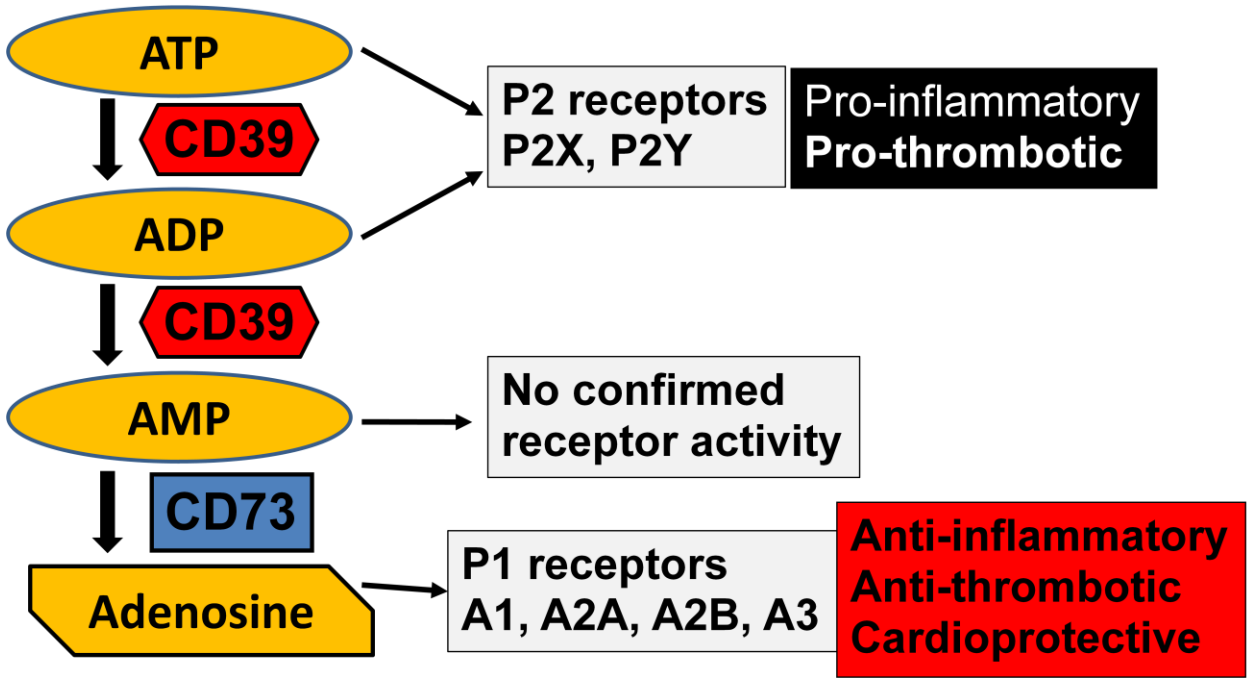
Table 1: Complete blood count results from bone marrow transplant and untreated groups

Parameter	WT (n=5)	CD39-TG (n=5)	WT → CD39-TG (n=6)	CD39-TG → WT (n=5)	p
White Blood Cells (K / µL)	12.6 ± 1.2	9.1 ± 1.7	12.0 ± 1.4	12.9 ± 2.2	ns
Red Blood Cells (K / µL)	11.2 ± 0.3	9.5 ± 1.0	9.6 ± 0.2	9.2 ±0.3	ns
Platelets (K / µL)	939.8 ± 172.8	1180.0 ± 180.3	862.0 ± 86.9	760.0 ± 61.7	ns

No significant statistical difference between groups, please see Figure 3 for a graphical representation of data.

FIGURES:

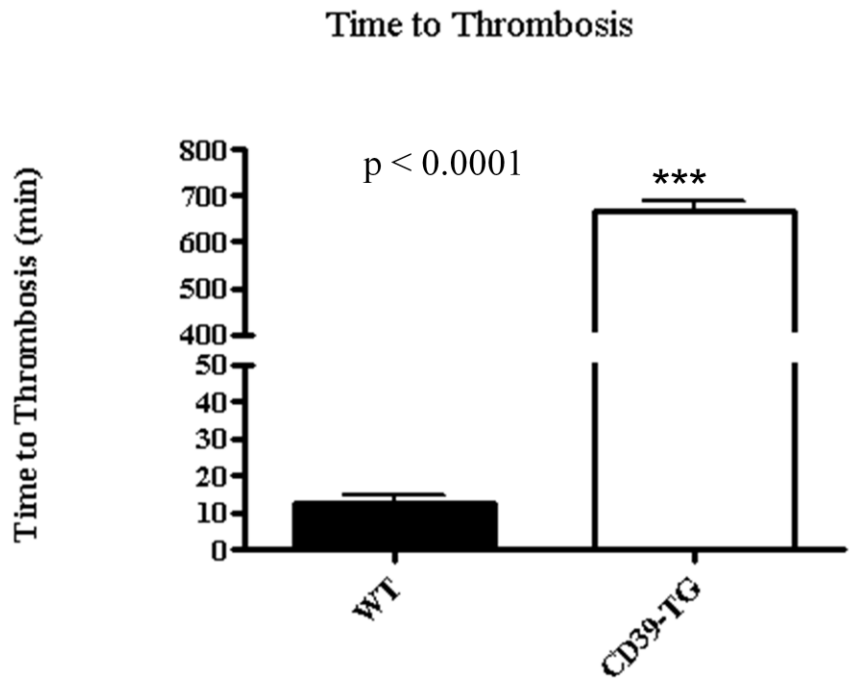
Figure 1:



Schematic of CD39/CD73 activity and purinergic receptor signaling overview.

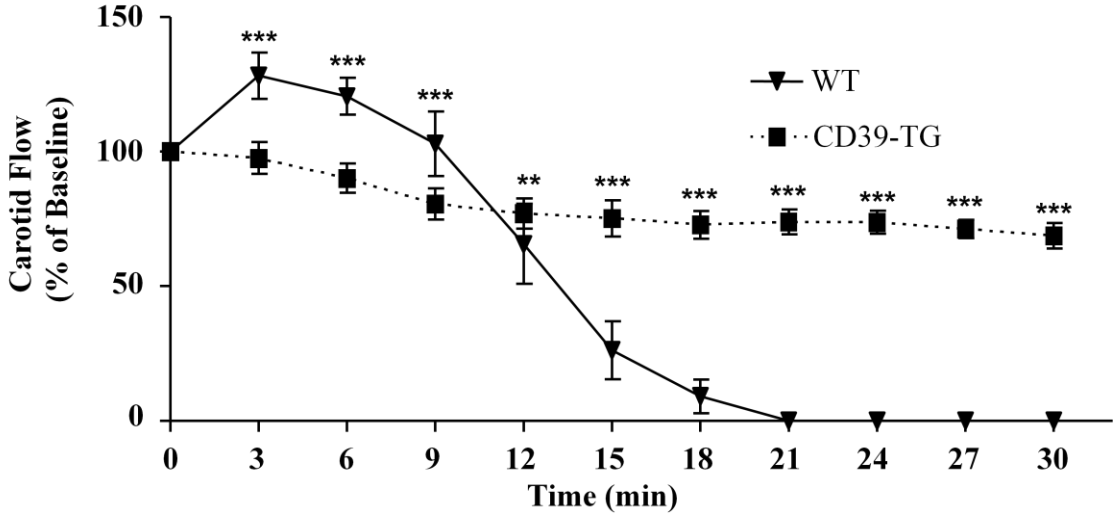
Figure 2:

2a:



Baseline times to thrombosis for WT (12.69 ± 2.17 min, n = 5) and CD39-TG (665.0 ± 24.5 min, n = 3)

2b:



Time plot showing percentage flow of initial following ferric chloride induced vessel injury (ferric chloride applied for 3 minutes starting at Time = 0) for WT and CD39-TG animals.

Figure 3:

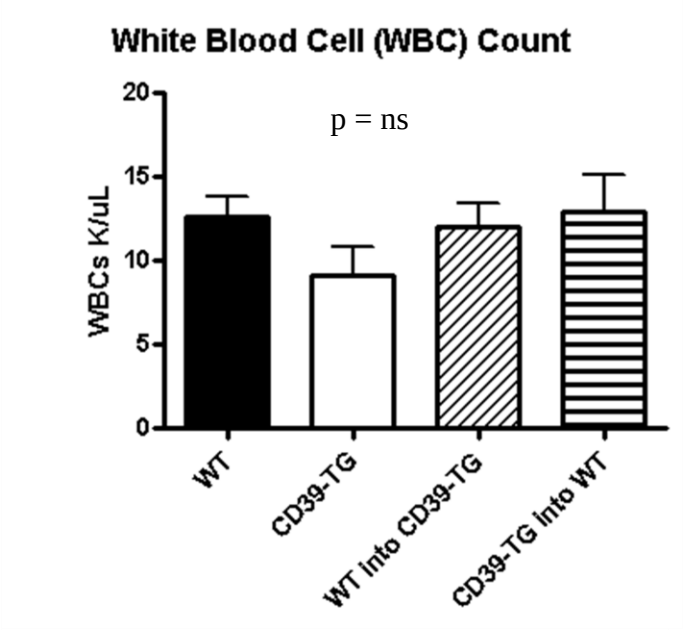


Figure 3a:
Graphical representation of White Blood Cell (WBC) counts.

WT baseline: 12.6 ± 1.2 K / µL, n = 5;
TG baseline: 9.1 ± 1.7 K / µL, n = 5;
WT into CD39-TG: 12.0 ± 1.4 K / µL, n = 6
CD39-TG into WT: 12.9 ± 2.2 K / µL, n = 5

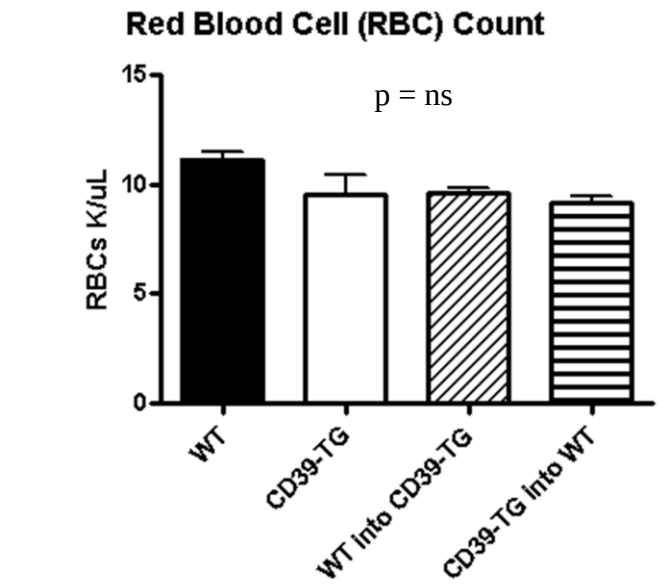


Figure 3b:
Graphical representation of Red Blood Cell (RBC) counts.

WT baseline: 11.2 ± 0.3 K / µL, n = 5;
TG baseline: 9.5 ± 1.0 K / µL, n = 5;
WT into CD39-TG: 9.6 ± 0.2 K / µL, n = 6
CD39-TG into WT: 9.2 ± 0.3 K / µL, n = 5

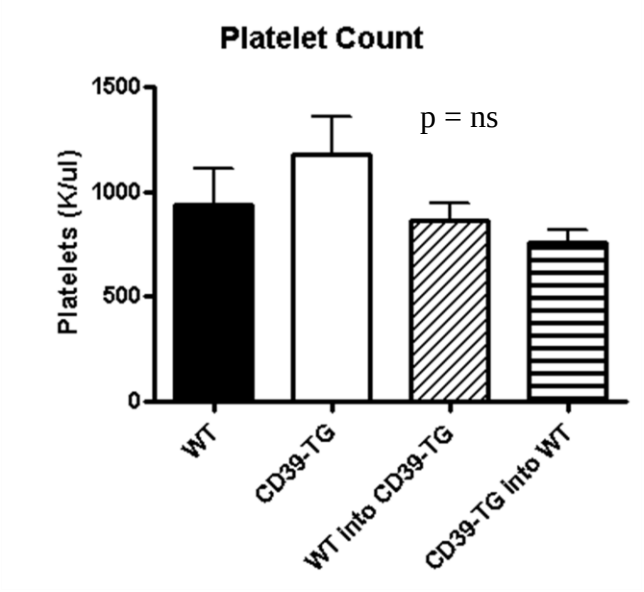


Figure 3c:
Graphical representation of Platelet cell counts.

WT baseline: 939.8 ± 172.8 K / µL, n = 5;
TG baseline: 1180.0 ± 86.9 7 K / µL, n = 5;
WT into CD39-TG: 862.0 ± 86.9 K / µL, n = 6
CD39-TG into WT: 760.0 ± 61.7 K / µL, n = 5

Figure 4:

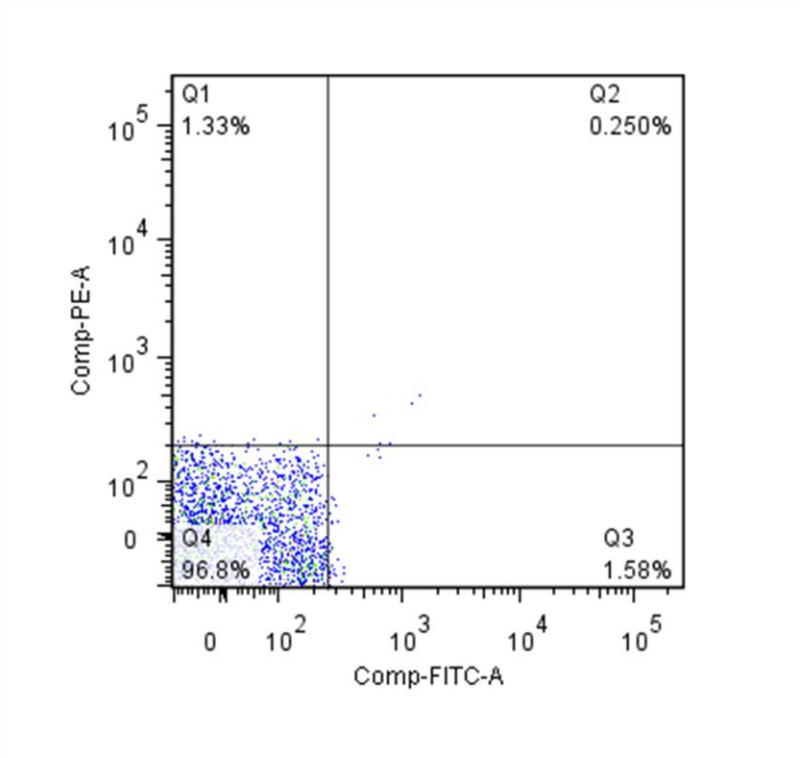


Figure 4a:
Pseudo-color plot showing leukocytes isolated from whole blood obtained from a WT animal which received CD39-TG bone marrow. These cells have not been incubated with any antibodies, they are serving as the negative control for the CD39-TG in WT group. 96.5 ± 0.3% of cells in Q4, n = 3.

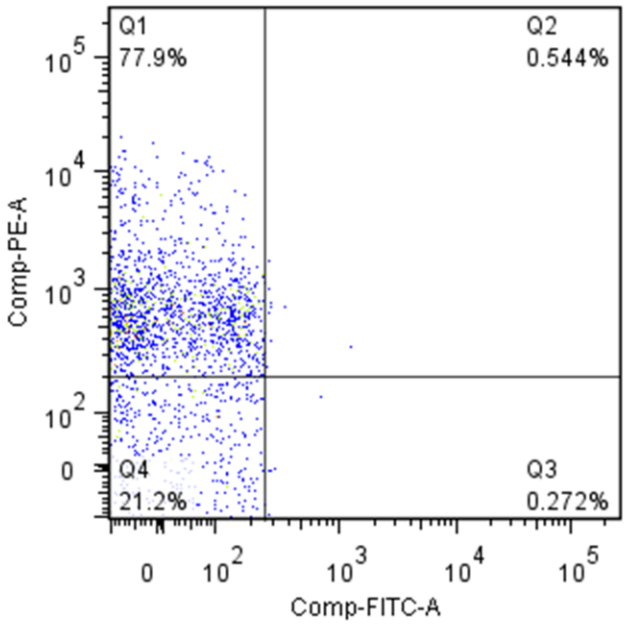


Figure 4b:
Pseudo-color plot showing leukocytes isolated from whole blood obtained from a WT animal which received CD39-TG bone marrow. These cells have been incubated with an anti-hCD39 antibody. 27.7 ± 3.3% of cells in Q4, 70.9 ± 3.5% in Q1, n = 3.

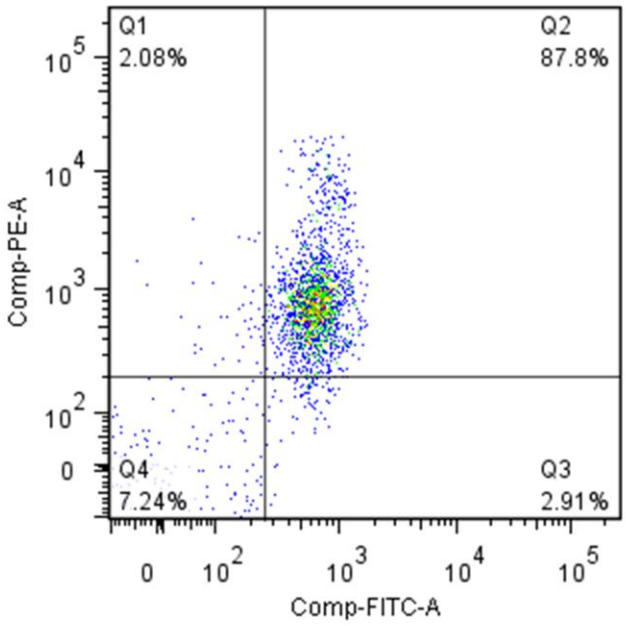


Figure 4c:
Pseudo-color plot showing leukocytes isolated from whole blood obtained from a WT animal which received CD39-TG bone marrow. These cells have been incubated with an anti-hCD39 antibody as well as an antibody to CD45. n = 3.

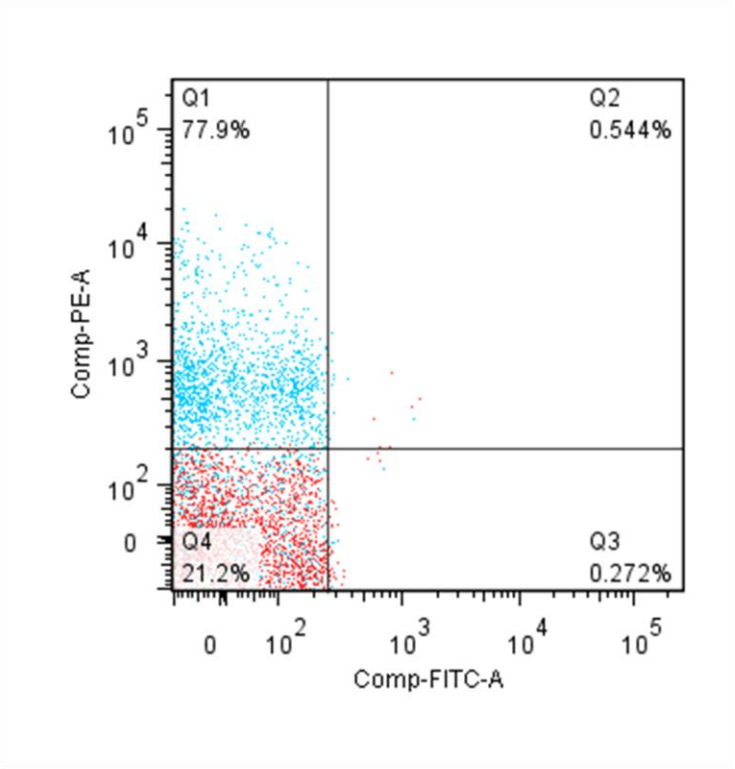


Figure 4d:
Pseudo-color plot showing an overlay of figures 4a and 4b. Unstained cells are shown in red and those incubated with anti-hCD39 are shown in blue.

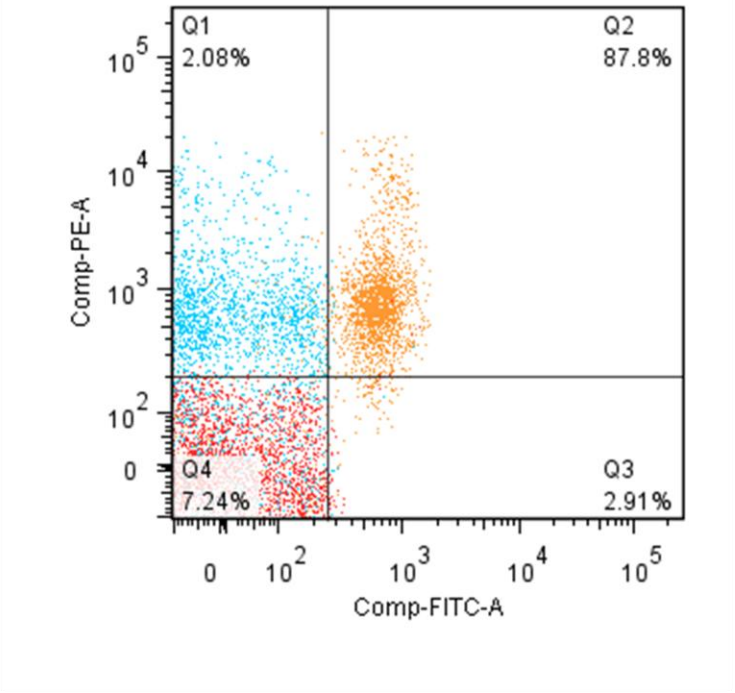


Figure 4e:
Pseudo-color plot showing an overlay of figures 4a-4c. Red are negative control cells, blue indicates cells stained with anti-hCD39, and orange shows cells stained with both anti-hCD39 and anti-CD45.

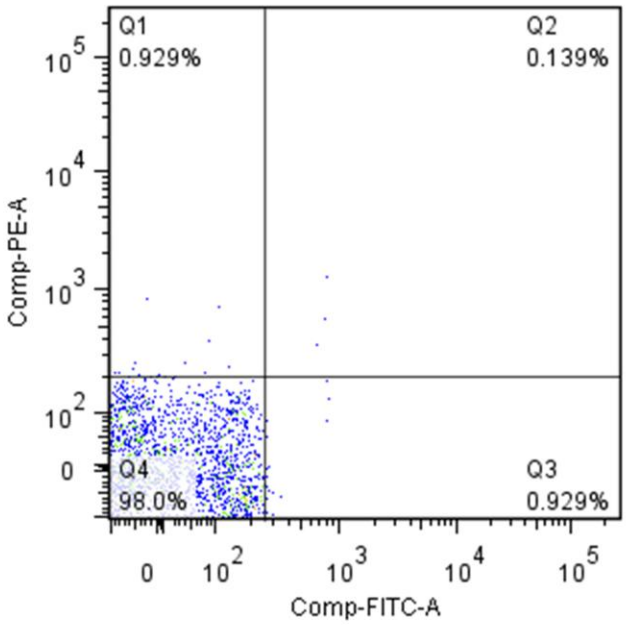


Figure 4f:
Pseudo-color plot showing leukocytes isolated from whole blood obtained from a CD39-TG animal which received WT bone marrow. These cells have not been incubated with any antibodies, they are serving as the negative control for the WT into CD39-TG group. 91.3 ± 6.1% of cells in Q4, n = 3.

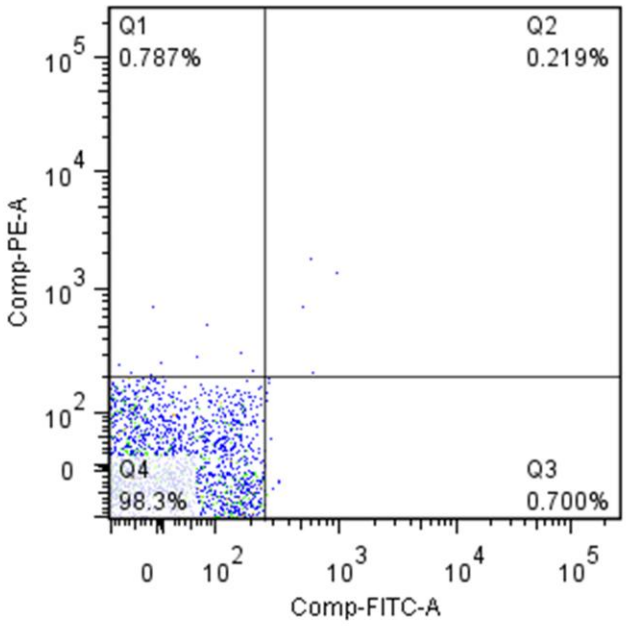


Figure 4g:
Pseudo-color plot showing leukocytes isolated from whole blood obtained from a CD39-TG animal which received WT bone marrow. These cells have been incubated with an anti-hCD39 antibody. 91.0± 7.0% of cells in Q4, 3.4 ± 2.5% in Q1, n = 3.

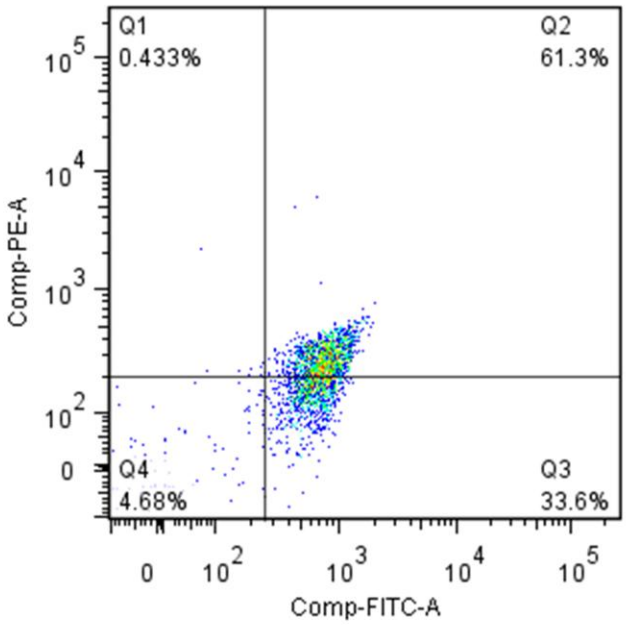


Figure 4h:
Pseudo-color plot showing leukocytes isolated from whole blood obtained from a CD39-TG animal which has received WT bone marrow. These cells have been incubated with an anti-hCD39 antibody as well as an anti-CD45 antibody. n = 3.

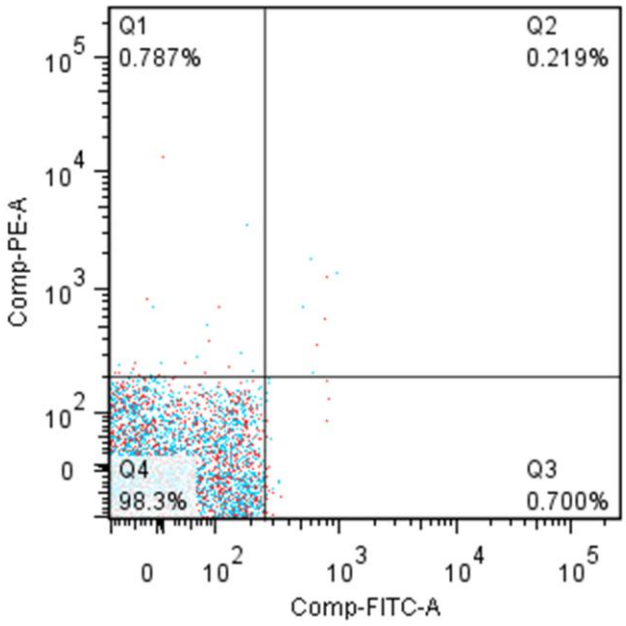


Figure 4i:
Pseudo-color plot showing an overlay of figures 4f and 4g. Unstained cells are shown in red and those incubated with anti-hCD39 are shown in blue.

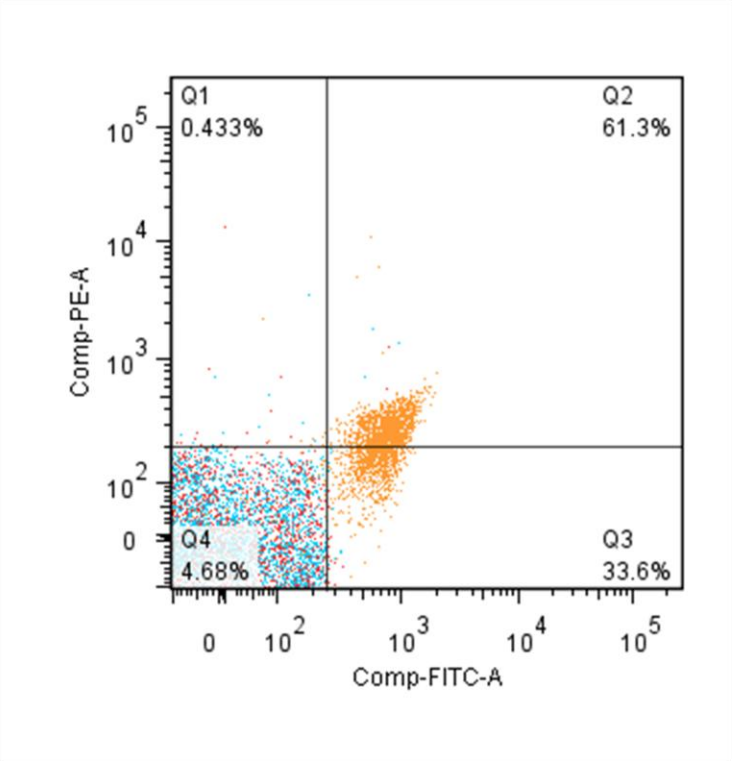


Figure 4j:
Pseudo-color plot showing an overlay of figure 4f – 4h. Unstained cells are shown in red, cells incubated with anti-hCD39 are shown in blue, and cells incubated with both anti-hCD39 and anti-CD45 are shown in orange.

Figure 5:

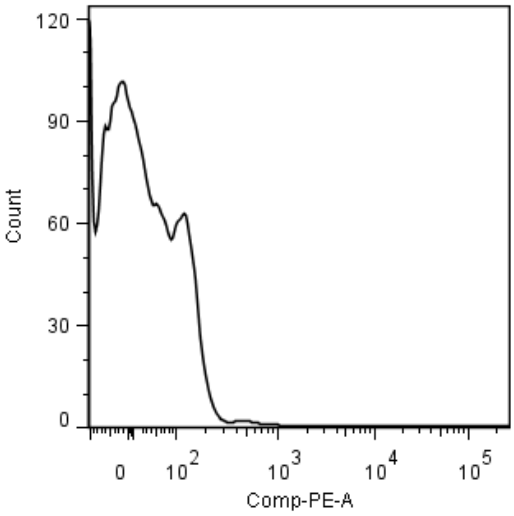


Figure 5a:
Histogram plot for leukocytes obtained from whole blood which has not been incubated with any antibodies. Geometric means: WT → CD39-TG: 7.2 ± 2.0, n = 3. CD39-TG → WT: 8.7 ± 1.3, n = 3, p = ns.

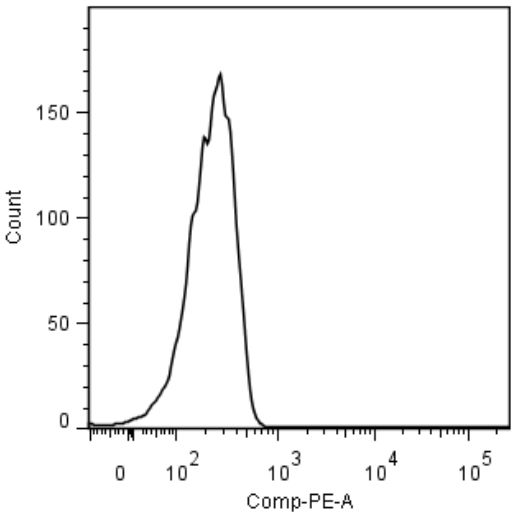


Figure 5b:
Histogram plot for leukocytes isolated from whole blood obtained from a CD39-TG animal which received WT bone marrow. Leukocytes have been stained with antibodies to hCD39 and CD45. Geometric mean: 183.0 ± 20.5, n = 3.

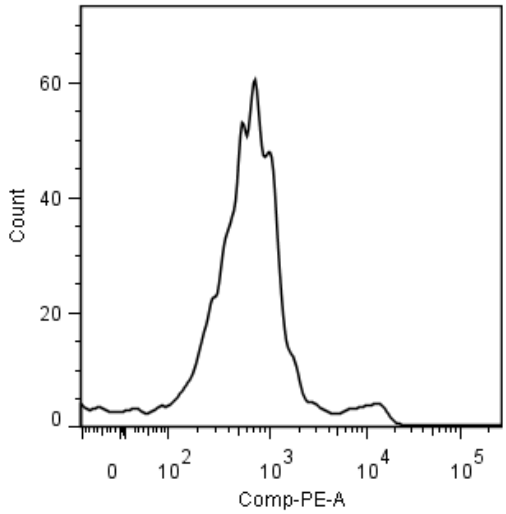


Figure 5c:
Histogram plot for leukocytes isolated from whole blood obtained from a WT animal which received CD39-TG bone marrow. Leukocytes have been stained with antibodies to hCD39 and CD45. Geometric mean: 522.7 ± 35.3, n = 3.

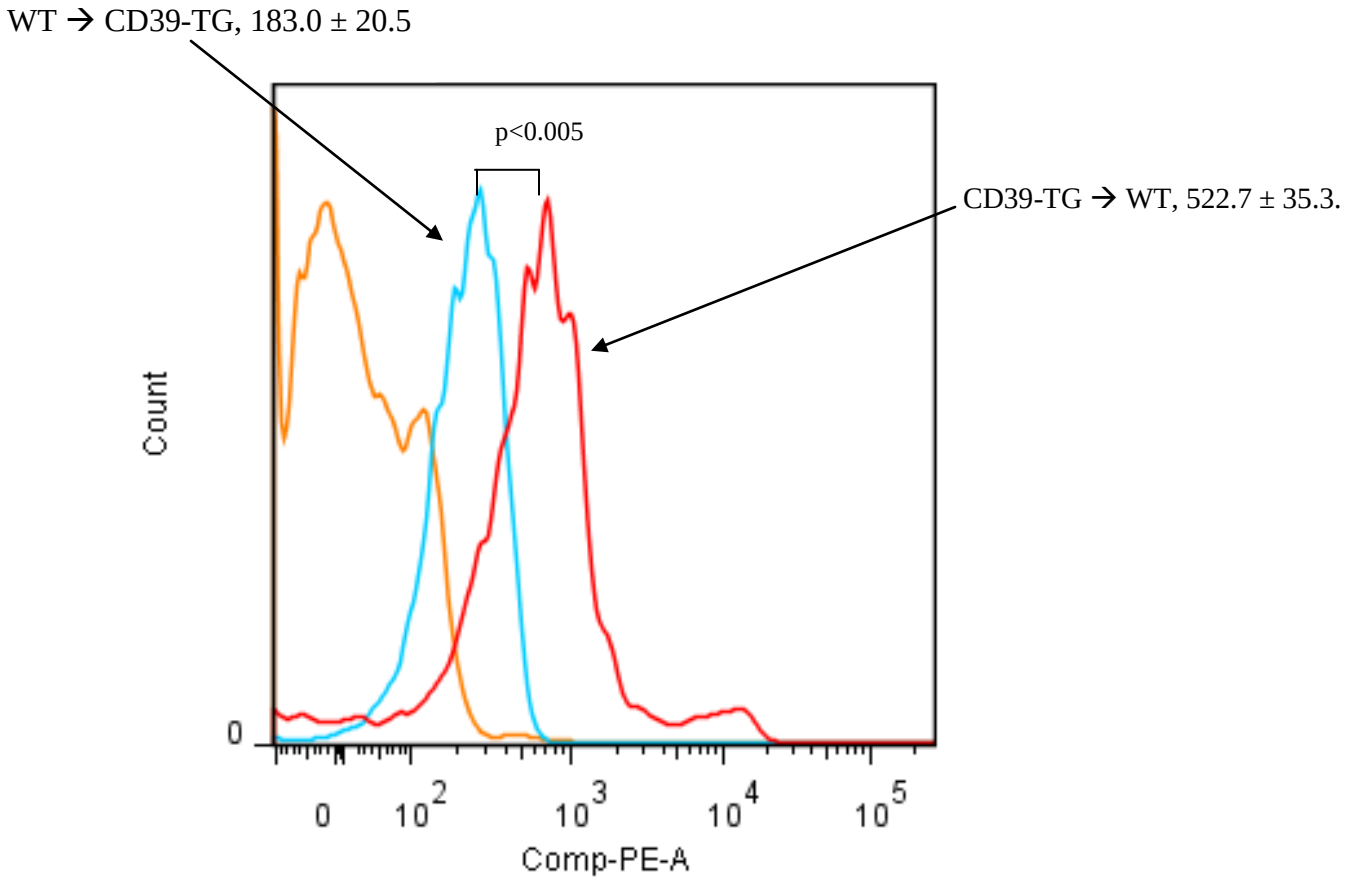
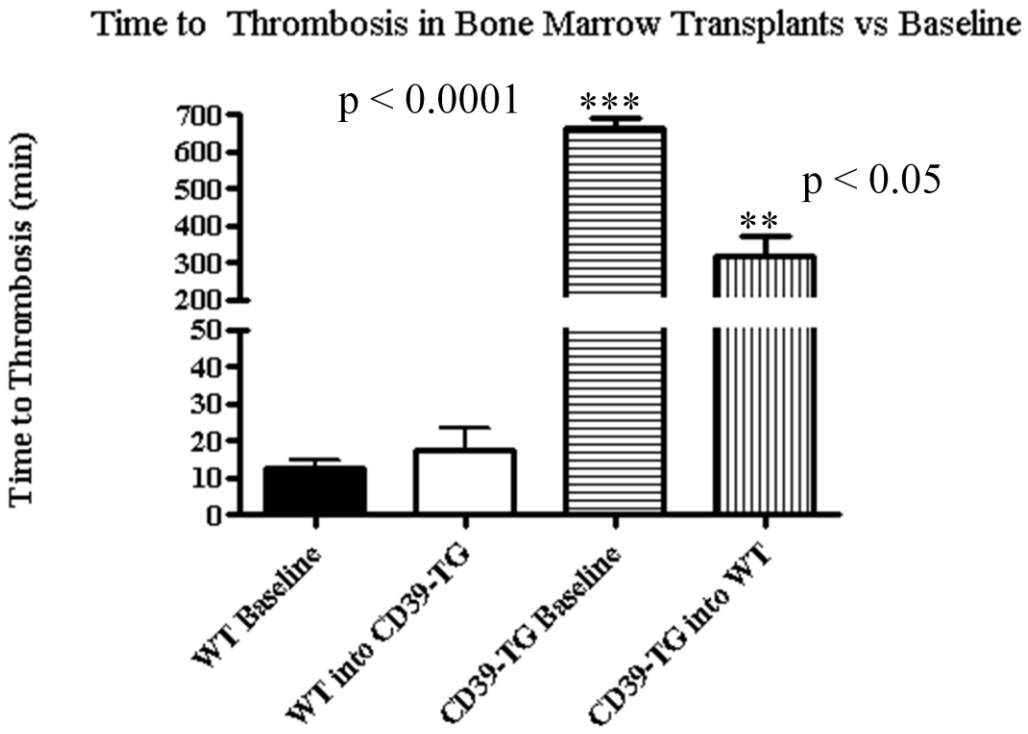


Figure 5d:
Histogram plot showing an overlay of figures 5a – 5c. Orange is the control baseline, blue depicts WT → CD39-TG, and red depicts CD39-TG → WT.

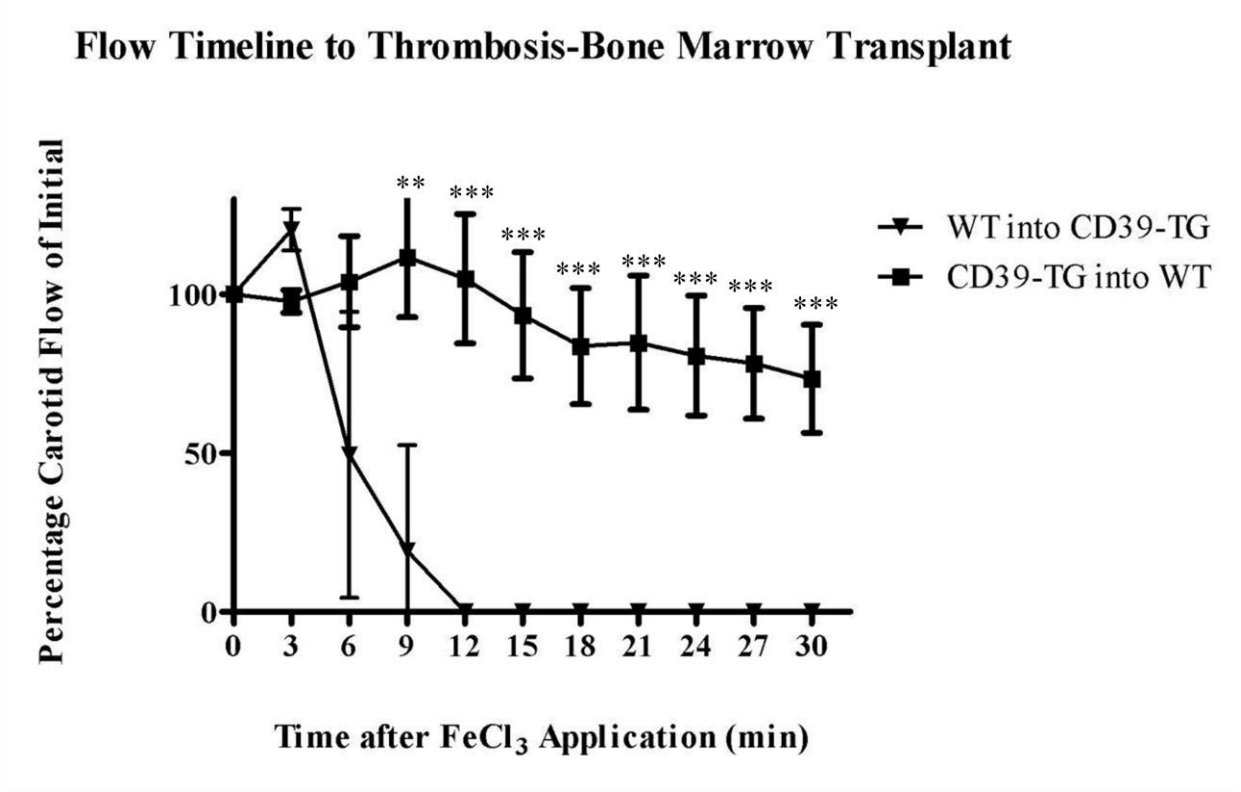
Figure 6:

6a:



Comparison of the times to thrombosis for bone marrow transplant (BMT) experiments versus baseline experiments. WT baseline: (12.69 ± 2.17 min, n = 5), WT into CD39-TG BMT: (12.28 ± 4.54, n = 4), CD39-TG baseline: (665.00 ± 24.50 min, n = 3), CD39-TG into WT BMT: (329.70 ± 68.15, n = 4).

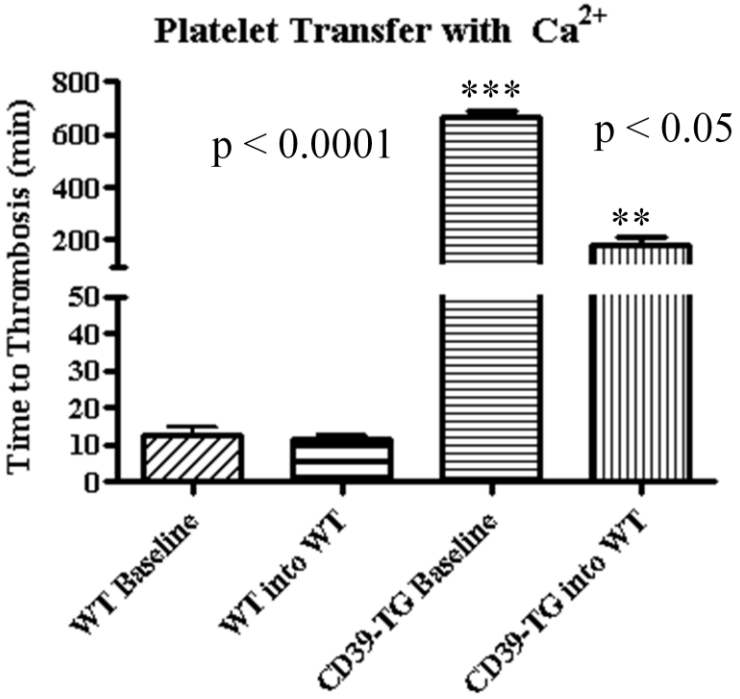
6b:



Time plot showing percentage flow of initial following ferric chloride induced vessel injury (ferric chloride applied for 3 minutes starting at Time = 0) for WT in CD39-TG and CD39-TG into WT bone marrow transplant animals.

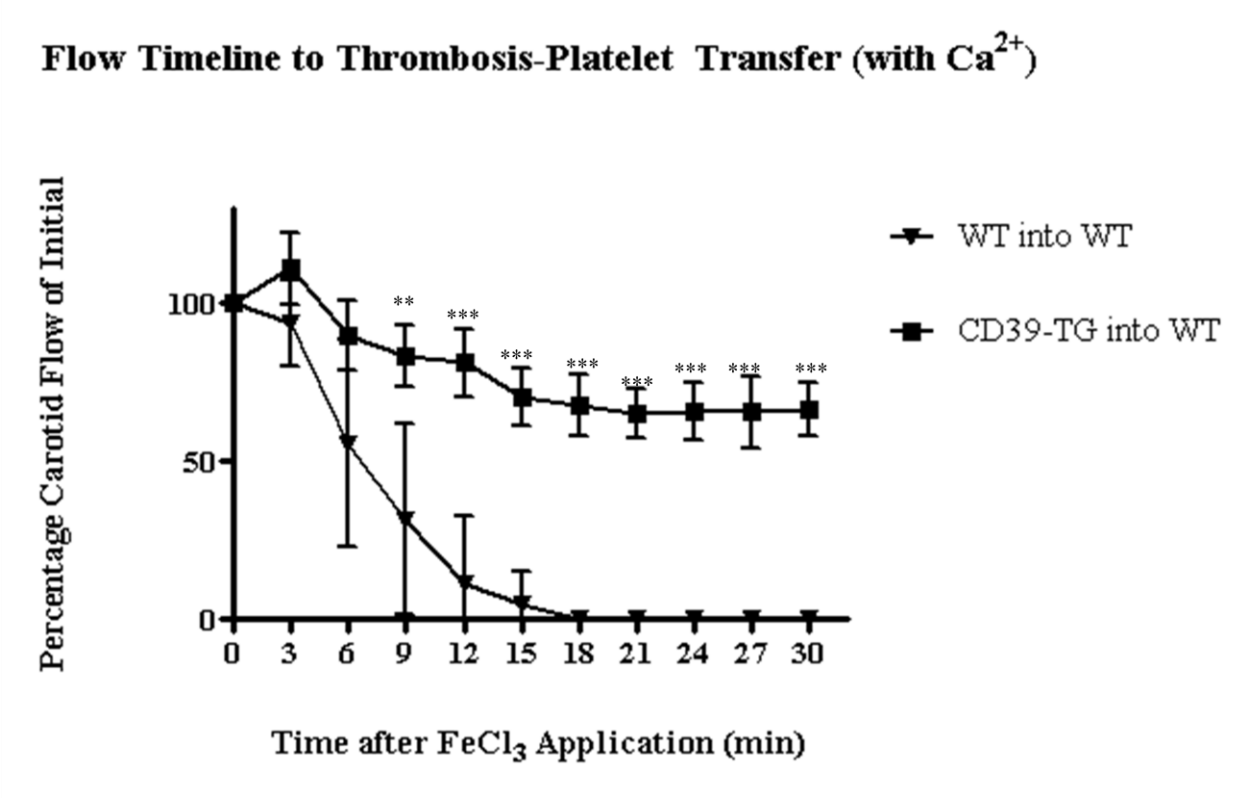
Figure 7:

7a:



Comparison of times to thrombosis between WT and CD39-TG baseline versus WT into WT and CD39-TG into WT platelet transfer experiments when Tyrode's buffer containing 1 mM calcium was used to isolate and purify platelets. WT baseline: (12.69 ± 2.17 min, n = 5), WT into WT: (11.21 ± 1.58, n = 5), CD39-TG baseline: (665.00 ± 24.50 min, n = 3), CD39-TG into WT: (179.90 ± 34.70 min, n = 5).

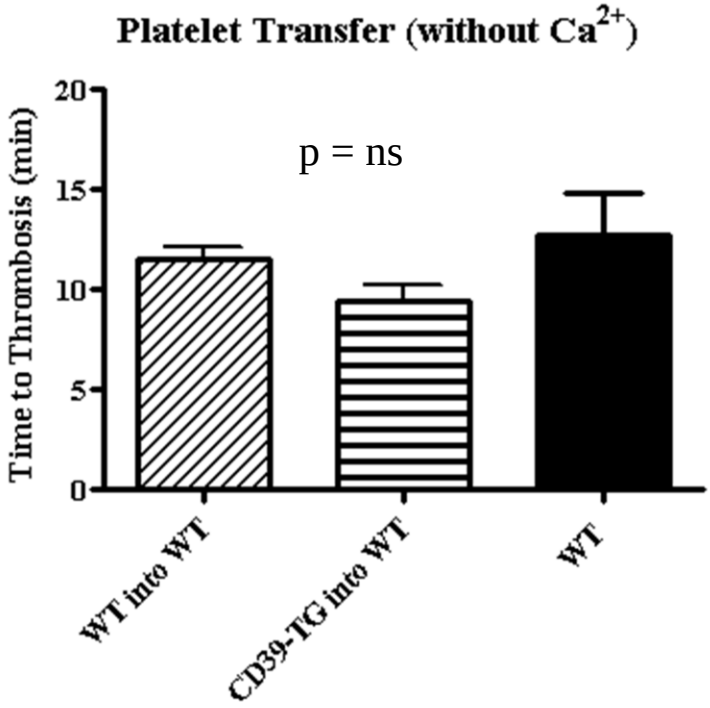
7b:



Time plot showing percentage flow of initial following ferric chloride induced vessel injury (ferric chloride applied for 3 minutes starting at Time = 0) for WT into WT platelet transfer and CD39-TG into WT platelet transfer experiments when buffer containing 1 mM calcium was used to isolate and purify platelets.

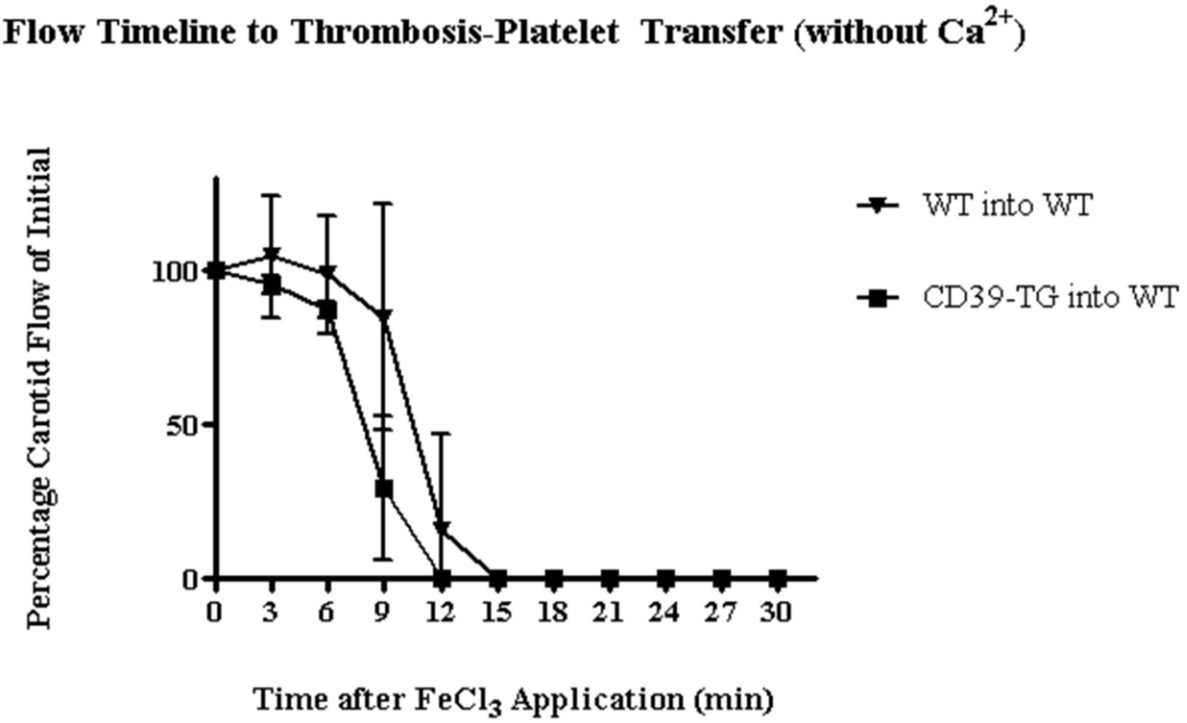
Figure 8:

8a:



Comparing the times to thrombosis between WT into WT platelet transfer, CD39-TG into WT platelet transfer and WT baseline when Tyrode's buffer void of calcium was used to isolate and purify platelets. No significant difference between groups. WT into WT: (11.53 ± 0.62 min, n = 4), CD39-TG into WT: (9.43 ± 0.83 min, n = 4), WT baseline: (12.69 ± 2.17 min, n =5).

8b:



Time plot showing percentage flow of initial following ferric chloride induced vessel injury (ferric chloride applied for 3 minutes starting at Time = 0) for WT into WT platelet transfer and CD39-TG into WT platelet transfer experiments when buffer void of calcium was used to isolate and purify platelts.

REFERENCES:

1. Kochanekm KD, Xu J, Murphy SL, Miniño AM, Kung H-C: Deaths: Preliminary Data for 2009. National Vital Statistics Reports, 2011, 59: 1-51

2. Born GVR: Aggregation of Blood Platelets by Adenosine Diphosphate and its Reversal, Nature, 1962, 194: 297-299

3. Kahner BN, Shankar H, Murugappan S, Prasad GL, Kunapuli SP: Nucleotide receptor signaling in platelets, Thromb Haemost, 2006, 4: 2317-2326

4. Freedman JE: Molecular regulation of platelet-dependent thrombosis, Circulation, 2005, 112: 2725-2734

5. Rolf MG, Brearley CA, Mahaut-Smith MP: Platelet Shape Change Evoked by Selective Activation of $P2X_1$ Purinoceptors with α-β-Methylene ATP, Thromb Haemost, 2001, 85: 303-308

6. Freedman JE: Molecular regulation of platelet-dependent thrombosis, Circulation 2005, 112: 2725-2734

7. Kaczmarek E, Koziak K, Sevigny J, Siegel JB, Anrather J, Beaudoin AR, Bach FH, Robson SC: Identification and characterization of CD39/vascular ATP diphosphohydrolase, J Biol Chem 1996, 271: 33116-33122

8. Koziak K, Sevigny J, Robson SC, Siegel JB, Kaczmarek E: Analysis of CD39/ATP diphosphohydrolase (ATPDase) expression in endothelial cells, platelets and leukocytes, Thrombosis and haemostasis 1999, 82: 1538-1544

9. Colgan SP, Eltzschig HK, Eckle T, Thompson LF: Physiological roles for ecto-5'-nucleotidase (CD73), Purinergic Signal 2006, 2: 351–360

10. Huttinger ZM, Milks MW, Nickoli MS, Aurand W, Long LC, Wheeler DG, Dwyer KM d'APice AJF, Robson SC, Cowan PJ, Gumina RJ: Ectonucleotide triphosphate diphosphohydrolase-1 (CD39) mediates resistance to occlusive arterial thrombus formation following vascular injury in mice, A J Path, 2012, in press

11. Dwyer KM, Robson SC, Nandurkar HH, Campbell DJ, Gock H, Murray-Segal LJ, Fisicaro N, Mysore TB, Kaczmarek E, Cowan PJ, d'Apice AJ: Thromboregulatory manifestations in human CD39 transgenic mice and the implications for thrombotic

disease and transplantation, J Clin Invest, 2004, 113: 1440-1446

12. Wang X, Xu L: An optimized murine model of ferric chloride-induced arterial thrombosis for thrombosis research, Thromb Res, 2005, 115: 95-100

13. Boisvert WA, Spangeberg J, Curtiss LK: Treatment of Severe Hypercholesterolemia in Apolipoprotein E-Defficient Mice by Bone Marrow Transplantation, J Clin Invest, 1995, 96: 1118-1124

14. Shapiro-Shelef M, Lin KI, Savitsky D, Liao J, Calame J: Blimp-1 is required for mainenance of long-lived plasma cells in the bone marrow, J Exp Med, 2005, 202: 1471-1476

15. Hechler B, Freund M, Alame G, Leguay C, Gaertner S, Cazenave JP, Petitou M, Gachet C: The Antithrombotic Activity of EP224283, a Neutralizable Dual Factor Xa Inhibitor/Glycoprotein IIbIIIa Antagonist, Exceeds That of the Coadministered Parent Compounds, J Pharmacol Exp Ther, 2011, 338: 412-420

16. Kowalska MA, Ratajczak MZ, Majka M, Jin J, Kunapuli S, Brass L, Poncz M: Stromal cell-derived factor-1 and macrophage-dervived chemokine: 2 chemokines that activate platelets, Blood, 2000, 96: 50-57

17. Zhang G, Thornton MA, kowalska MA, Sachis BS, Feldman M, Poncz M, McKenzie SE, Reilly M: Localization of distal regulatory domains in the megakaryocyte-specific platelet basic protein/platelet factor 4 gene locus, Blood, 2001, 98: 610-617

18. Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MAS, Storb RF, Hammond WP: Evidence for Circulating Bone Marrow-Derived Endothelial Cells, Blood, 1998, 92: 362-367

19. Ackroyd JF: Role of Platelets in Coagulation, Thrombosis and Haemostasis, with some Observations of Platelet Dysfunction, including Thrombasthemia, Bristish Medical Bulletin, 1955, 11: 21-27

20. Gleen JR, White AE, Johnson AJ, Fox SC, Myers B, Heptinstall S: Raised levels of CD39 in leucocytosis result in marked inhibition of ADP-induced platelet aggregation via rapid ADP hydrolysis, Platelets, 2008, 19: 59-69

21. von Brühl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, Khandoga A, Tirniceriu A, Coletti R, Köllnberger M, Byrne RA, Laitinen I, Walch A, Brill A, Pfeiler

S, Manukyan D, Braun S, Lange P, Riegger J, Ware J, Eckart A, Haidari S, Rudelius M, Shulz C, Echtler K, Brinkmann V, Schwaiger M, Preissner KT, Wagner DD, Mackman N, Engelmann B, Massberg S: Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis is mice in vivo, J Exp Med, 2012, 209: 819-835

22. Hyman MC, Petrovic-Djergovic D, Visovatti SH, Liao H, Yanamadala S, Bouïs D, SU EJ, Lawrence DA, Broekman MJ, Marcus AJ, Pinsky DJ: Self-regulation of inflammatory cell trafficking in mice by the leukocyte apyrase CD39, J Clin Invest, 2009, 109: 1136-1149

23. Michelson A, Barnard M, Krueger L, Valeri C, Furman M: Circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selection: studies in baboons, human coronary intervention and human acute myocardial infarction, Circulation, 2001, 104: 1533-1537

24. Marcus AJ, Broekman MJ, Drosopoulos JHF, Olson KE, Islam N, Pinsky DJ, Levi LR: Role of CD39 (NTPDase-1) in Thromboregulation, Cerebroprotection, and Cardioprotection, Semin Thromb Hemost, 2005, 31: 234-246

25. Sturgeon SA, Jones C, Angus JA, Wright CE: Adaptation of the Folts and electrolytic methods of arterial thrombosis for the study of anti-thrombotic molecules in animals, J Pharmacological and Toxicological Methods, 2006, 53: 20-29

26. Bush LR, Shebuski RJ: In vivo models of arterial thrombosis and thrombolysis. Faseb Journal, 1990, 4: 3087-3098

27. Folts JD, Schafer AI, Loscalzo J, Willerson JT, Miller JE: A perspective on the potential problems with aspirin as an antithrombotic agent: A comparison of studies in an animal model with clinical trials, J Am Coll Cardio, 1999, 23: 295-303

28. Savi P, Herbert JM: Clopidogrel and Triclopidine: $P2Y_{12}$ Adenosine Diphosphate-Receptor Antagonists for the prevention of Atherothrombosis, Semin Thromb Hemost, 2005, 31: 174-183

29. Malek LA, Kisiel B, Spinewak M, Grabowski M, Fillipiak KJ, Kostrzewa G, Huczek Z, Ploski R, Opolski G: Coexisting polymorphisms of P2Y12 and CYP2C19 genes as a risk factor for persistent platelet activation with clopidogrel, Circ J, 2008, 72: 1165-1169